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Detection of T-DNA transfer to plant cells by *A. tumefaciens* virulence mutants using agroinfection

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Summary. To test whether virulence mutants of *Agrobacterium tumefaciens* are capable of promoting T-DNA transfer into plant cells, a tandem array of Cauliflower Mosaic Virus (CaMV) DNA was cloned between T-region border sequences on a wide host range plasmid and introduced into various virulence mutants. The resulting strains were used to infect *Brassica rapa* cv. Just Right. This assay, recently referred to as agroinfection, is based on the appearance of viral symptoms following transfer of T-DNA to plant cells, and is shown to be at least 100 times more sensitive in detecting T-DNA transfer than tumour formation. Mutants in the loci *vir* A, B and G, which were avirulent on turnip, failed to induce virus symptoms. Of the two *vir* D mutants tested, neither induced tumours, but one was capable of inducing virus symptoms. Mutants in *vir* E, C and F, which induced respectively no, small and normal tumours on turnip, all induced virus symptoms.

Key words: *A. tumefaciens* – Agroinfection, virulence genes – T-DNA transfer – Cauliflower mosaic virus

Introduction

Two regions on the Ti plasmid of *Agrobacterium tumefaciens* are essential for plant tumour induction; the T-region and the virulence (vir)-region. All other Ti plasmid encoded functions can be inactivated or deleted without affecting the tumour-inducing capacity (for recent reviews see Hille et al. 1984a; Nester et al. 1984; Schell et al. 1984).

During tumourigenesis, the T-region harbouring the oncogenes is transferred to plant cells and integrated into the nuclear genome (Thomashow et al. 1980). Transfer of T-DNA from the bacterium into the plant cell and integration into the nuclear genome does not depend on the oncogenes themselves, but on the presence of border sequences flanking the T-region on the Ti plasmid. The T-region border sequences alone are sufficient to allow transfer and integration into the plant nuclear genome of foreign DNA sequences inserted between them (Zambryski et al. 1983).

The *vir*-region has never been detected (in part) in transformed plant cells (Thomashow et al. 1980). It encodes trans-acting products which are functional within the bacterium (Hille et al. 1982; Klee et al. 1982) and supposedly mediate early steps in tumourigenesis, including conditioning of plant cells, T-DNA intermediate formation, transfer of T-DNA from bacterium to the plant cell and possibly the integration of T-DNA into the plant genome (Kado 1984; Hille et al. 1985; Koukoliková-Nicola et al. 1985). Upon contact between the bacterium and plant cells expression of *vir* genes is highly stimulated by plant signal molecules (Okker et al. 1984; Stachel et al. 1986). Seven loci have been identified in the *vir*-region of the octopine Ti plasmid, referred to as *virA, B, G, C, D, E* and *F* (Iyer et al. 1982; Klee et al. 1983; Hille et al. 1984b; Hooykaas et al. 1984; Stachel et al. 1986). For virulence, not all these loci are absolutely needed by the bacterium. Some loci seem to determine the plant host range and/or the size of the tumour induced. Thus far, specific functions have only been suggested for the gene products of the *virA* and *virG* loci. The *virA* protein(s) is thought to be involved in the initial recognition and/or intracellular transport of the plant signal molecule (Stachel et al. 1985b). The *virG* protein might then be activated allosterically by this signal molecule/*virA* complex so as to activate *vir* transcription by interacting with *vir* gene promoter sequences (Stachel et al. 1985b).

Recently, the plant cell transformation system of *A. tumefaciens* has been combined with the biological activity of cloned viral DNA (Grimsley et al. 1986; Gardner and Knauf 1986). Thus, local introduction of a DNA sequence by *A. tumefaciens* can be assayed visually by spread of virus or viroid throughout the plant. This assay system has been named agroinfection (Grimsley et al. 1986).

In this paper we have addressed the question of whether virulence mutants of *A. tumefaciens* are capable of transferring T-DNA to plant cells using the agroinfection assay. To this end, cells in a wound site of turnip plants were transformed by *A. tumefaciens* strains carrying *vir*-functions and a tandem copy of infectious cloned CaMV-DNA on separate plasmids and plants were scored for the appearance of viral symptoms. We have also analysed the sensitivity of the agroinfection assay as compared to tumour induction.

Materials and methods

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Tables 1 and 2. Conjugations between *Escherichia coli* and *Agrobacterium tumefaciens* were performed as previously reported (Hille et al. 1982). Plasmid

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Table 1. Bacterial strains and plasmids

Strain plasmid	Relevant phenotype	Source
E. coli strain		
HB101	Str^r, RecA$^-$	J. Collins
GJ23 (pGJ28, pR64drd11)	Km^r, Tc^r, Sm^r	Van Haute et al. (1983)
MM294 (pRK2013)	Km^r	Ditta et al. (1980)
HP3435	Rif^r	H. Pannekoek
A. tumefaciens strains		
C58 (pGV3850)	Rif^r, Ap^r, vir$^+$, onc$^-$	Zambryski et al. (1983)
LBA288 (−)	Rif^r, vir$^-$, onc$^-$	P. Hooykaas
LBA958 (pTiC58)	Rif^r, vir$^+$, onc$^+$	P. Hooykaas
LBA1010 (pTiB6)	Rif^r, vir$^+$, onc$^+$	P. Hooykaas
LBA4404 (pAL4404)	Rif^r, Sm^r, vir$^+$, onc$^-$	Hoekema et al. (1983)
Plasmids		
pCa305	Ap^r, Sp^r	Grimsley et al. (1986)
pAGS121b	Ap^r, Tc^r	Van den Elzen et al. (1985)

pCTW300 was transferred from *E. coli* strain HB101, with the help of *E. coli* strain MM294 (pRK2013), to different *A. tumefaciens* virulence mutants. *A. tumefaciens* transconjugants were selected on minimal medium plates solidified with 1.8% Difco agar (Hooykaas 1979) containing kanamycin (100 μg/ml) and tetracycline (2.5 μg/ml). After 3 days of incubation at 29° C, individual colonies were tested on minimal medium plates for the presence of all antibiotic resistance markers: carbenicillin (Cb), 100 μg/ml (5 μg/ml in the case of strain LBA4404); kanamycin (Km), 100 μg/ml; spectinomycin (Sp), 250 μg/ml; streptomycin (Sm), 500 μg/ml; tetracycline (Tc), 2.5 μg/ml. *A. tumefaciens* transconjugants, harbouring pCTW300, were then purified twice on minimal medium containing kanamycin and tetracycline. These bacteria were subsequently used for inoculation on turnip. Bacteria from the same plate were also used in a three point cross with *E. coli* strains MM294 (pRK2013) and HP3435 in order to transfer pCTW300 from *A. tumefaciens* to *E. coli* strain HP3435 (selection on LB medium supplemented with 20 μg/ml rifampicin and 15 μg/ml tetracycline incubated at 37° C). By this procedure, the integrity of plasmid pCTW300 was ascertained in the different *A. tumefaciens* virulence mutants used for plant inoculation.

DNA manipulations. All DNA manipulations (plasmid DNA isolation, restriction endonuclease digestion and gel electrophoresis) were performed according to Maniatis et al. (1982).

Inoculation of turnips with A. tumefaciens. A sterile wooden toothpick was dipped into a colony of the strain to be tested and then used to puncture the tuber of a 5-week-old turnip plant (*Brassica rapa* cv. Just Right). Turnips were propagated in an insect free green house and examined for the appearance of tumours and virus symptoms. The first symptoms of CaMV infection on turnip appear as local lesions on one or a few leaves, whereas at a later stage of infection leaves show strong vein-clearing and some deformations.

Table 2. T-DNA transfer to turnip by *A. tumefaciens*

Vir locus affected	Plasmid pCTW300 present in *A. tumefaciens* strain number	Inoculated on turnip	
		tumour formation	CaMV symptoms
vir$^+$ onc$^+$	LBA958, LBA1010	+	+
vir$^+$ onc$^-$	LBA4404	−	+
vir$^-$ onc$^-$	LBA288	−	−
*vir*A$^-$	A1016, A1007	−	−
*vir*B$^-$	LBA1512, LBA1516, LBA1532, A2002, A2003, A2005, A1000, A1024, A1074, A1009, A1012, A1018, A1023, A1031, A1035, A1040, A1010	−	−
*vir*G$^-$	A348::vir G363	−	−
*vir*C$^-$	LBA1551, A1034	+/−	+
*vir*D$^-$	A1021	−	−
	A1028	−	+
*vir*E$^-$	LBA1514, A1002, A1026	−	+/−
*vir*F$^-$	LBA1517	+	+

All mutants have been tested in two separate experiments. In the first experiment, 5 turnip plants were inoculated per mutant strain and in the second experiment 12 turnip plants per mutant strain. Thus, each *A. tumefaciens* strain was tested on 17 turnip plants. Plants were scored 6–7 weeks after inoculation. In the scoring for tumour induction, + indicates normal tumour size; +/− reduced tumour size, and − no clear tumour induction. In the scoring for CaMV symptoms, + indicates more than 14 out of 17 plants infected, +/− indicates 2–4 infected plants, and − indicates no virus infected plants. However, in some exceptionel cases we found among a series of non-infected plants, one virus infected plant. In these cases we retested the corresponding *A. tumefaciens* mutant strain on ioturnip plants and found it to be unable to induce virus symptoms. All *A. tumefaciens* strains with an LBA number were obtained from Dr. P. Hooykaas and are described in Hooykaas et al. 1984, and all A numbers from Dr. E.W. Nester and described in Garfinkel and Nester 1980 and Stachel et al. 1985a. For the position of the mutations in the different mutants, see Fig. 3

Mechanical inoculation of turnips with plasmid DNA. One or two leaves of five-week-old turnip plants were inoculated with 10 μg plasmid DNA (pCTW300 or pCa305) using carborundum as an abrasive. Plants were scored for virus symptoms 4 weeks after inoculation.

Results

Construction of plasmid pCTW300

For the construction of an *A. tumefaciens* compatible "CaMV-plasmid", we made use of two plasmids, pCa305 (Grimsley et al. 1986) which contains a 1.3 tandem genome of CaMV and cannot replicate in *A. tumefaciens*, and pAGS121b (Van den Elzen et al. 1985), a wide host-range plasmid carrying T-region border sequences. As both plasmids share homology at the ampicillin-resistance region, homologous recombination allows integration of the 1.3 copy of CaMV-DNA between the T-region border se-

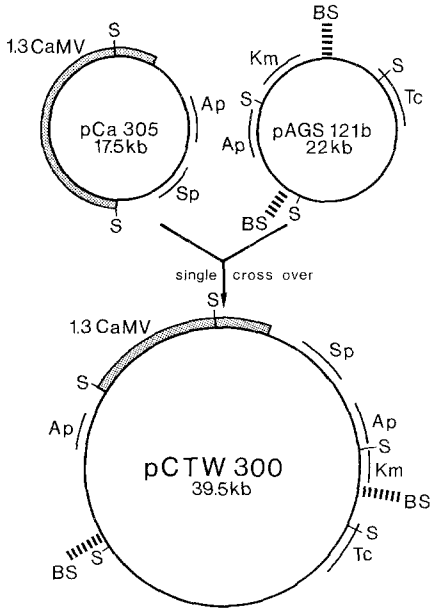

Fig. 1. Construction of an *A. tumefaciens* compatible "CaMV plasmid". Plasmid pCa305, which harbours a 1.3 tandem copy of CaMV and can only be maintained in *E. coli*, was conjugatively transferred from *E. coli* strain GJ23 (pGJ28, pR64drd11, pCa305) to *A. tumefaciens* strain LBA288 (pAGS121b). *A. tumefaciens* transconjugants harbouring the cointegrate plasmid pCTW300, in which plasmid pCa305 has recombined into plasmid pAGS 121b by homologous recombination, were selected on minimal medium supplemented with spectinomycin. The physical structure of pCTW300 was verified by digesting the plasmid DNA with *SalI* followed by agarose gel electrophoresis. Tc, tetracycline resistance; Ap, ampicillin resistance; Sp, spectinomycin resistance; Km, kanamycin resistance; BS, T-region border sequence; S, restriction endonuclease *SalI* site

quences. Plasmid pCa305 was introduced by conjugation into an *A. tumefaciens* strain, harbouring plasmid pAGS121b, and transconjugants containing the cointegrate plasmid (pCTW300) were selected on minimal medium using spectinomycin as a marker. Restriction endonuclease analysis revealed the structure of this *A. tumefaciens* compatible "CaMV-plasmid" as shown in Fig. 1. To test the biological activity of plasmid pCTW300, purified plasmid DNA was rubbed onto turnip leaves and plants were scored for the appearance of virus symptoms. As with the control plasmid pCa305, plants became systemically infected within 4 weeks post inoculation, demonstrating that pCTW300 contains all the features essential for virus production.

T-DNA transfer by A. tumefaciens virulence mutants

As the approach of using virus symptoms to detect T-DNA transfer to plant cells is not dependent on tumour formation and most probably also not on T-DNA integration into the plant genome, we addressed the question whether *vir*-mutants of *A. tumefaciens* were capable of transferring T-DNA to plant cells. Plasmid pCTW300, containing a 1.3 tandem copy of CaMV between T-region border sequences, was conjugatively transferred to *A. tumefaciens* strains containing mutations in one of the seven identified *vir*-loci of an octopine Ti plasmid; *virA, B, G, C, D, E* and *F* (see Materials and methods and Fig. 3). Transconjugants were then inoculated onto turnips and plants were scored for both tumour formation and systemic CaMV infection, 6 weeks following inoculation. The results are presented in Table 2 and an example of this agroinfection assay is shown in Fig. 2. Mutants in the *virA, B* and *G* loci, which are known to be avirulent on all plant species tested thus far, including turnip, failed to induce CaMV infection on turnips, suggesting that these mutants do not transfer T-DNA to plant cells at a detectable level. A similar result was obtained with *virD* mutant A1021. However, another *virD* mutant, in which the mutation has been identified in the 3′ part of the locus (A1028), did induce virus symptoms on turnip, although tumour formation was virtually invisible. This indicates that T-DNA transfer to turnip cells does occur in this *virD* mutant.

Mutants in *virC, E* and *F* display a host-dependent effect in that they induce tumour formation to a variable extent on some plant species, but not on others. Thus, it has been reported that a functional *virF* locus is essential for tumour formation on tomato and tobacco (Hooykaas et al. 1984) but not on turnip. Consequently, virus symptoms can be expected to occur on turnip following inoculation with *A. tumefaciens virF* mutants which also harbour plasmid

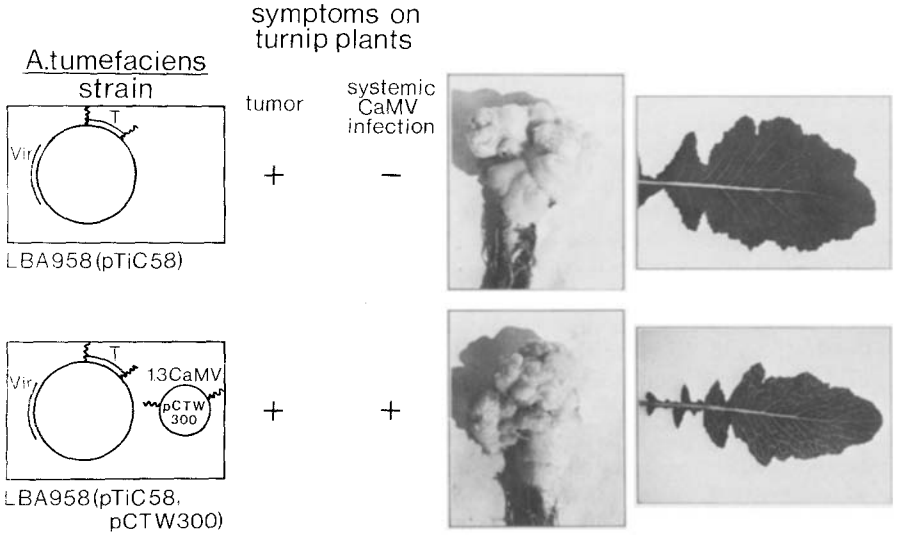

Fig. 2. *A. tumefaciens* directed T-DNA transfer to turnip. Upon inoculation in the tuber, a wild-type *A. tumefaciens* strain LBA958 (pTiC58) induces a tumour on turnip whereas the leaves remain normal. In the presence of the *A. tumefaciens* compatible "CaMV-plasmid" pCTW300, strain LBA958 (pTiC58, pCTW300) induces a tumour on turnip and, through systemic spread, CaMV-symptoms on the leaves

Table 3. Sensitivity of detecting T-DNA transfer to turnip using *A. tumefaciens*-directed CaMV infection

Inoculum *A. tumefaciens* strain(s)	Number of plants tested	Number of systemically infected plants after		
		4 weeks	7 weeks	9 weeks
C58 (pGV3850)	10	0	0	0
C58 (pGV3850::pCa305)	10	10	10	10
Mixture of C58 (pGV3850::pCa305)/C58 (pGV3850) at a ratio of				
10^{-1}	10	10	10	10
10^{-2}	10	3	9	10
10^{-3}	10	0	9	10
10^{-4}	10	0	5	9

Turnip plants were inoculated in the tuber with *A. tumefaciens* strains C58(pGV3850), C58(pGV3850::pCa305) or mixtures of both as indicated, using an inoculum of approximately 10^8 agrobacteria per wound site

pCTW300. As shown in Table 2 this indeed was the case. Similarly, inoculation of turnip with *virC* mutants containing pCTW300 gave rise to both tumour formation (although the size of the tumour was reduced) and systemic virus infection.

Mutants in *virE* have been reported to induce distinct tumours only on *Kalanchoë tubiflora* (Otten et al. 1984). Indeed, no tumour formation occurred on turnip in our studies. However, 10%–20% of the plants inoculated with the three different *virE* mutants containing pCTW300 showed virus symptoms, indicating that all three *virE* mutants do direct T-DNA transfer to plant cells.

Sensitivity of the agroinfection assay

For wounds with a fixed size, tumour size is proportional to the amount of oncogenic bacteria used to induce the tumour (Schilperoort 1969). When using an inoculum of agrobacteria in which 10% of the agrobacteria are oncogenic, a very small tumour develops. No visible tumour formation occurs upon inoculation with a 1% oncogenic *A. tumefaciens* population (Lippincott and Lippincott 1969; Schilperoort 1969; J. Hille, unpublished). As the assays of detecting T-DNA transfer by tumour formation and systemic CaMV infection are essentially different, we set up experiments to determine the sensitivity of the CaMV assay. Plasmid pCa305 (see Fig. 1) was conjugatively transferred to *A. tumefaciens* strain C58 and inserted between the T-region border sequences of the non-oncogenic Ti plasmid vector pGV3850 via homologous recombination. The resulting strain C58 (pGV3850::pCa305) and the control strain C58(pGV3850) were grown separately, mixed in different ratios and inoculated onto turnip plants using approximately 10^8 agrobacteria per wound site. Plants were scored for the appearance of systemic infection at 4, 7 and 9 weeks following inoculation. As shown in Table 3, virus production became evident at all ratios tested. Even at a ratio of 10^{-4} C58(pGV3850::pCa305)/C58(pGV3850) virus symptoms could be detected 9 weeks post-inoculation in 9 out of 10 plants inoculated. These results demonstrate that *A. tumefaciens*-directed CaMV infection provides a sensitive assay for the detection of T-DNA transfer to plant cells that is at least 100 times more sensitive than tumour induction.

Discussion

In this work we have used systemic CaMV infection as an assay system to analyse *vir* mutants of an octopine strain of *A. tumefaciens* for their ability to promote transfer of T-DNA into turnip. This so-called agroinfection assay (Grimsley et al. 1986) bypasses tumour formation, acquired antibiotic resistance or hormone autotrophy as criteria for successful transformation of plant cells by *A. tumefaciens*. Neither the genetic background of the *A. tumefaciens* strains, [whether C58 as in LBA958 or Ach5 as in LBA4404], nor the kind of Ti plasmid present, [nopaline as in LBA958 (pTiC58) or octopine as in LBA1010 (pTiB6)] had a significant effect on the ability to induce systemic CaMV infection on turnip (see Table 2).

An important aspect of agroinfection is its high sensitivity, which allows the detection of hitherto undetectable levels of T-DNA transfer from *A. tumefaciens* to plant cells. Here, we have shown that an inoculum of agrobacteria in which 0.01% of the agrobacteria contain the 1.3 tandem genome copy of CaMV between T-region border sequences still efficiently induce systemic CaMV infection on turnip. As compared to tumour formation, agroinfection is at least 100 times more sensitive in detecting T-DNA transfer to plant cells.

Although we cannot rule out completely the possibility that the expression of the transferred CaMV-plasmic occurs only following its integration into the host genome, we find this possibility rather unlikely in view of the ability of purified plasmids pCa305 and pCTW300 to induce systemic infection upon mechanical inoculation (see also Grimsley et al. 1986). This implies that merely the presence of T-DNA carrying the 1.3 tandem copy of the CaMV genome in plant cells should be sufficient to allow systemic infection. Thus, if T-DNA is transferred to plant cells and in some stage present as double-stranded DNA, virus infection can be expected to occur.

Vir A, B and G

Our studies demonstrate that *virA, B* and *G* mutants are not only avirulent but also fail to induce virus symptoms as determined by the agroinfection assay, suggesting that these mutants do not transfer T-DNA to plant cells at a detectable level. As these *vir* loci are highly conserved at the nucleotide sequence level among different Ti-plasmids (Engler et al. 1981) it is suggested that the gene products of the loci *virA, B* and *G* constitute a set of conserved functions that are indispensable for transferring T-DNA from the bacteria into the plant cells.

The suggested functions for the *virA* and *virG* loci, namely transmitting a plant signal inside *A. tumefaciens* and inducing expression of *vir* loci (Stachel et al. 1985b) are in accordance with our results. Mutants in such loci are not expected to transfer T-DNA to plant cells, since the *vir* genes can not be properly activated.

Recently, an agroinfection system has been described for tomato, using potato spindle tuber viroid (PSTV) as the infectious agent (Gardner and Knauf 1986). In testing three *virA* and three *virB* mutants for transfer of T-DNA

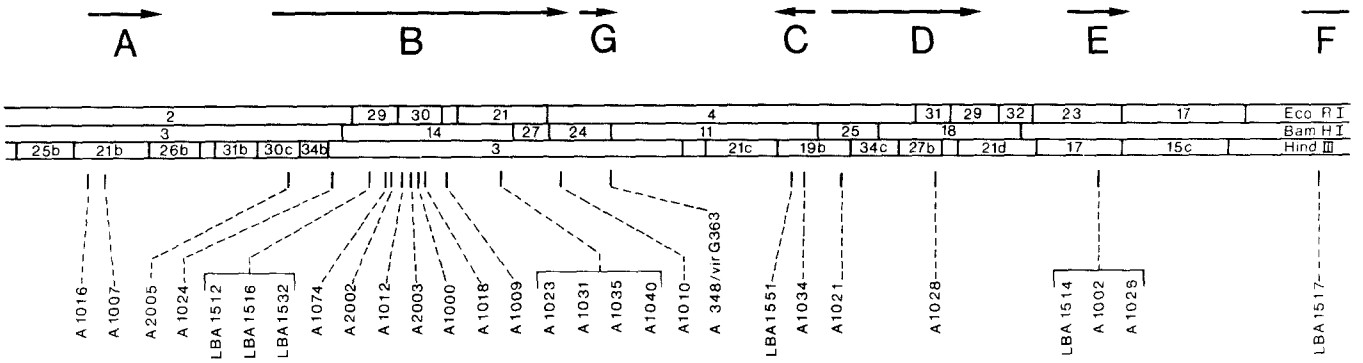

Fig. 3. Position of insertion mutations in *vir* mutants. The location of insertion mutations in the *Vir* region has been indicated and marked by the corresponding strain numbers (see Table 2). The positions and directions of transcription indicated for *vir* loci is according to Stachel et al. 1986 and Hooykaas et al. 1984

to plant cells, these authors arrived at the same conclusion, namely that these mutants do not transfer T-DNA detectably to plant cells.

VirD

The *virD* locus of a nopaline Ti plasmid has been sequenced (Hagiya et al. 1985). It was shown that *virD* might be an operon containing four distinct genes. Since the nopaline and octopine Ti plasmids are largely homologous to each other in this region, it seems reasonable to assume that the octopine *virD* locus also consists of four genes. We have tested a *virD* mutant containing a mutation in the first gene of the operon (A1021, see Fig. 3) and were unable to detect either tumour formation or virus symptoms on turnip plants. However, the inability of this mutant to transfer T-DNA to plant cells might be due to a polar effect of the transposon Tn*5* insertion and thus not reflect the role of the other genes of the operon. Another mutation in *virD* (in mutant A1028, see Fig. 3) is located in the 3′ part of the locus. This mutant barely gave rise to tumours, but in the agroinfection assay efficiently induced virus symptoms. Thus, mutant A1028 does transfer T-DNA to plant cells but apparently at a low frequency. Gardner and Knauf (1986) also tested a *virD* mutant, in which the position of the mutation is close to that of mutant A1028. However, they did not obtain viroid infection in the agroinfection assay using this mutant. This then might be explained if the mutation in that mutant is located in a different gene of the *virD* locus.

VirC, E and F

Mutations in the loci *VirC, E* and *F* seem to determine the plant host range for tumour induction and the size of the tumour. In the presence of plasmid pCTW300, all mutants in *virC, E* and *F* were found capable of inducing systemic CaMV infection on turnip, though their tumourigenicity varied considerably. In case of *virF* and *virC* mutants, tumour formation was obvious, whereas no tumour growth developed upon inoculating turnips with *virE* mutants. In the case of *virE* mutants, transfer of T-DNA could be detected by the agroinfection assay, but the frequency was low as only 10%–20% of the inoculated plants revealed virus symptoms. Moreover, the time of occurrence of virus symptoms, 6–7 weeks after inoculation, indicates a low frequency of T-DNA transfer from *A. tumefaciens*. Gardner and Knauf (1986) showed that *virE* mutants do transfer T-DNA to tomato and suggested that the *virE* gene product(s) are active in steps which lead to integration of T-DNA into the plant genome, following transfer of T-DNA into the plant cell. However our results on turnip show a low frequency of T-DNA transfer into plant cells with *virE* mutants and only because of the higher sensitivity of the agroinfection assay compared to tumour formation, can T-DNA transfer of *virE* mutants be observed.

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